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### Permalink

<https://escholarship.org/uc/item/28h7371x>

### Journal

Scientific reports, 4(1)

### ISSN

2045-2322

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### Publication Date

2014-05-01

### DOI

10.1038/srep04963

Peer reviewed



## OPEN

## SUBJECT AREAS:

BIOLOGICAL  
ANTHROPOLOGY

GLYCOMICS

GLYCOBIOLOGY

Received  
30 December 2013Accepted  
24 April 2014Published  
16 May 2014

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# Glycosylated proteins preserved over millennia: N-glycan analysis of Tyrolean Iceman, Scythian Princess and Warrior

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An improved understanding of glycosylation will provide new insights into many biological processes. In the analysis of oligosaccharides from biological samples, a strict regime is typically followed to ensure sample integrity. However, the fate of glycans that have been exposed to environmental conditions over millennia has not yet been investigated. This is also true for understanding the evolution of the glycosylation machinery in humans as well as in any other biological systems. In this study, we examined the glycosylation of tissue samples derived from four mummies which have been naturally preserved: – the 5,300 year old “Iceman called Oetzi”, found in the Tyrolean Alps; the 2,400 year old “Scythian warrior” and “Scythian Princess”, found in the Altai Mountains; and a 4 year old apartment mummy, found in Vienna/Austria. The number of N-glycans that were identified varied both with the age and the preservation status of the mummies. More glycan structures were discovered in the contemporary sample, as expected, however it is significant that glycan still exists in the ancient tissue samples. This discovery clearly shows that glycans persist for thousands of years, and these samples provide a vital insight into ancient glycosylation, offering us a window into the distant past.

The health and lifestyles of ancient humans remain a fascination as they provide insight into our own current conditions. The discovery of the world’s oldest natural ice mummy “Oetzi or Iceman” in 1991 has been of great interest to both the general public and the scientific community. The Iceman is already the subject of approximately one hundred publications in the past twenty years<sup>1–10</sup>. Studies have been conducted to analyze his origin<sup>11–16</sup>, cause of death<sup>17,18</sup>, mummification processes<sup>19</sup>, his tattoos<sup>20</sup> and even his last meal(s)<sup>21</sup>. Many others less known natural mummies have been discovered spanning a period over several thousand years. Mummies were found in Siberia of the Scythians, a long forgotten civilization that lived in currently southern Russia over two thousand years ago. These mummies are well preserved including hair and intricately tattooed bodies. However there is little information available in the literature about the “Scythian Warrior” and the “Scythian Princess”. Even recent mummies have been found. Mummies found in abandoned apartments from over 4 years provide examples.

Natural mummies spanning thousands of years offer a unique opportunity to examine ancient biochemical processes that took place in our early ancestors. Natural mummies are often formed as products of extreme conditions such those found in glaciers or deserts<sup>22</sup>. Unlike preserved mummies, such as the Egyptian mummies, natural mummies have not been exposed to chemical preservation processes thereby allowing the tissues to degrade naturally. The presence of these samples as well as the development of new and sensitive analytical tools to examine them have the potential to provide us with a wealth of new biological and chemical information.

One of the best ways to examine the health status of a living organism is to monitor changes in the proteome. Although histological investigations and ATR-IR spectroscopy demonstrated a high degree of protein preservation<sup>19</sup>, there have been surprisingly few studies on the proteomic analysis of the iceman<sup>23</sup>. Even less are studies



**Table 1 | Detailed sample information of fresh tissue and four natural mummies**

Sample ID	Sex	Estimated age	Estimated age of the corpse
<b>Iceman</b> <b>Scythian</b> <b>Warrior</b>	Male	46 y	5,300 y
	A Male	25 ± 5 y	2,400 y
	B		
	C		
<b>Scythian</b> <b>Princess</b>	A Female	25 ± 5 y	2,400 y
	B		
<b>Apartment</b> <b>Fresh Tissue</b>	Male	47 y	4 y
	Male	35 y	Fresh

related to post-translational modifications (PTMs), which moderate the activity of most eukaryotic proteins. The major types of PTM are phosphorylation, acetylation, glycosylation and methylation<sup>24,25</sup>. Glycosylation is unique among the PTMs in that it has complicated structures and is responsible for protecting the polypeptide backbone. It is also known that glycosylation is altered in many diseases, and hence has been the subject of many studies for its potential as diagnostic and prognostic biomarkers. There have been no studies of post-translational modifications of proteins with regard to whether they can withstand thousands of years of exposure. While the rapid dephosphorylation of phosphoproteins in human tissue has been discussed in the literature<sup>26</sup>, there are similarly no reports on what happens to glycosylation post-mortem. Therefore, little is known regarding glycosylated proteins and glycan structures in both post-mortem human samples and samples which have been preserved for thousands of years under ambient conditions. This study shows the N-glycan profile of four unique, naturally mummified samples from three different time points (4 y, 2,400 y and 5,300 y). We were able to identify the world's oldest glycans in the 5,300 year old iceman specimen. Moreover, distinct correlations between samples from the different time points were observed, showing that not only the number of glycans, but their size and complexity decrease over time.

## Results and Discussion

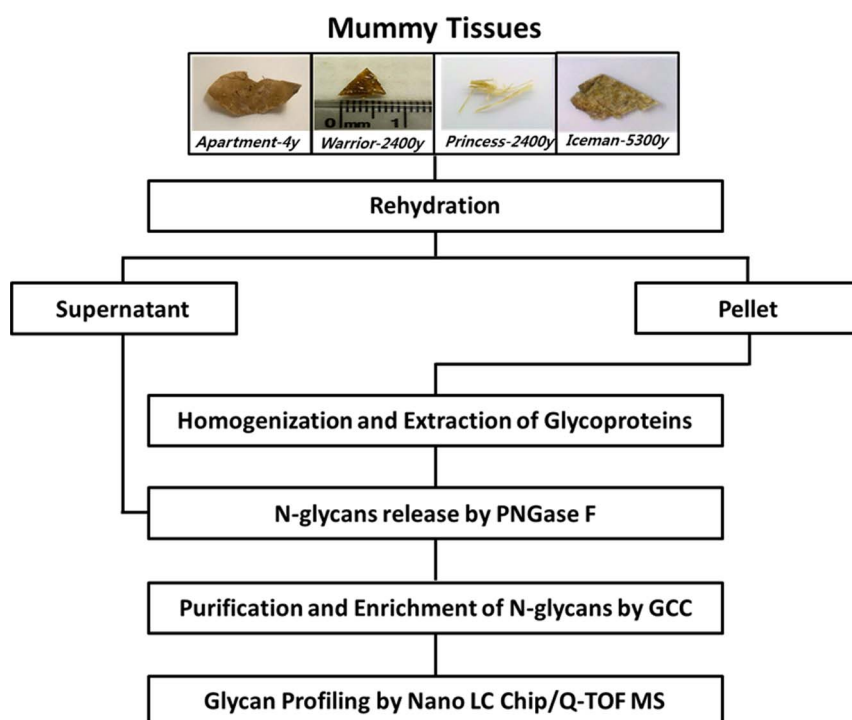
N-glycans were profiled from eight naturally mummified human tissues ranging from 4 to 5,300 years old, with fresh tissue as reference. Table 1 lists the detailed sample information including sample origin and the estimated age of the mummy samples. It was only possible to acquire multiple samples from the Scythian warrior and the Scythian princess.

An initial concern was whether there would be sufficient levels of oligosaccharides present in the mummy tissue samples for detection by mass spectrometry. In addition, there was the possibility that exposure to ambient conditions may have modified the glycan structures. To address these concerns, a streamlined method was developed to minimize sample handling and manipulation while analyzing the glycans in their original states.

The experimental procedure from tissue rehydration to glycan analysis is shown in Figure 1. The mummy tissues required hydration (36 hours) prior to glycan extraction because the samples were stiff and dry. The rehydrated tissues were centrifuged and then divided into pellet and supernatant portions in order to retain the greatest amount of proteins. After grinding the pellet with an extraction solution, the supernatant of the ground tissues was combined with the original supernatant from the rehydration step. N-glycans were enzymatically released by Peptide-N-Glycosidase F (PNGase F) from both the supernatant and the remaining pellet portion, separately. The released glycans were enriched by solid phase extraction using a porous graphitized carbon (PGC) cartridge. Purified glycans were then separated on a PGC microfluidic liquid chromatography chip and analyzed by mass spectrometry (MS).

We examined both N-linked and O-linked glycans, but O-glycans were not detected in the mummy samples. The absence of O-glycans can be explained by the fact that N-glycans are significantly more abundant in human glycoproteins and may mask the analysis<sup>27</sup>. Additionally, O-glycans are typically relatively short and may be more readily degraded by microorganisms such as bacteria<sup>28,29</sup>.

We also examined glycans from fresh human tissue sample, both as a reference point and to gain a better understanding of potential post-mortem processes and to observe potential evolutionary aspects



**Figure 1 | Experimental strategy to profile glycans from mummy tissue.**



Table 2 | Summary of abundant N-glycan found in fresh tissue and four natural mummies

Sample ID		# of Compositions	# of Compounds	Abundant Glycan Structures <sup>†</sup>
<b>Fresh Tissue</b>		69	230	
<b>Apartment (4 y)</b>		48	90	
<b>Scythian Warrior (2,400 y)</b>	A	24	72	
	B	19	64	
	C	47	175	
	D	42	148	
<b>Scythian Princess (2,400 y)</b>	A	6	16	
	B	6	18	
<b>Iceman (5,300 y)</b>		13	20	

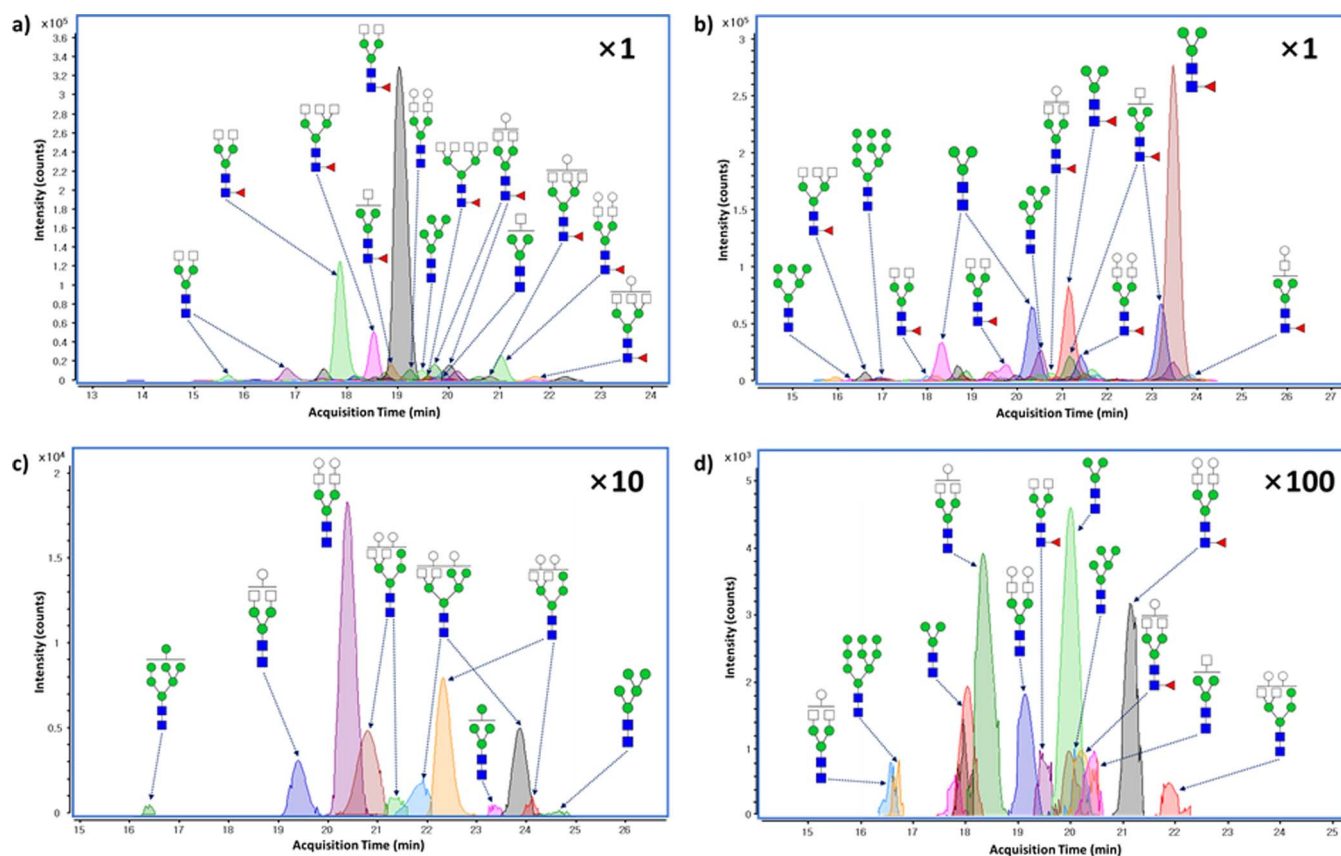
<sup>†</sup>Circle (white), hexose; circle (green), mannose; square (white), N-acetyl hexosamine; square (blue), N-acetyl glucosamine; triangle (red), fucose; diamond (purple), N-acetyl neuraminic acid.

of glycans that have been isolated from ancient mummy samples. Supplemental Figure 1 shows the overlay extracted ion chromatogram (EIC) of the most abundant N-glycans isolated from the fresh tissue extract. Sixty-nine different N-glycans were assigned based on accurate mass in the fresh tissue sample with the most common compositions corresponding to  $[\text{Hexose}(\text{H})_a : \text{N-acetyl hexosamine}(\text{HN})_b : \text{Fucose}(\text{F})_c : \text{Sialic acid}(\text{SA})_d]$  ( $a = 3-9$ ,  $b = 2-7$ ,  $c = 0-5$ , and  $d = 0-3$ ) (Supplemental Table 1). The majority of the glycan abundances consisted of bi-antennary mono-fucosylated complex type N-glycans corresponding to the ions  $m/z$  1786.643  $[\text{H}_5 : \text{HN}_4 : \text{F}_1]$ ,  $m/z$  1827.677  $[\text{H}_4 : \text{HN}_5 : \text{F}_1]$  and bi-antennary di-sialylated  $m/z$  2222.783  $[\text{H}_5 : \text{HN}_4 : \text{SA}_2]$ .

**Glycan profiles of natural mummies.** Four natural human mummies consisting of three ancient ice mummies - the 5,300 year old “Oetzi”, the 2,400 years old “Scythian warrior”, the 2,400 year old “Scythian princess”, and a contemporary 4 year old “apartment mummy” - were processed as described in the experimental

section. While the samples were partitioned and treated separately, the results were combined in order to provide a comprehensive picture of glycosylation.

N-glycans found in all four mummy samples were analyzed by nano HPLC-Chip/Q-TOF MS, with the results summarized in Table 2. Representative EICs of the most abundant glycans are shown in Figure 2 with their putative structures. In the contemporary apartment mummy, we found approximately 48 N-glycan compositions consisting of mainly bi-antennary mono-fucosylated complex type (Figure 2a). Many glycans found in the apartment mummy matched those found in the fresh human tissue sample. Figures 2b and 2c show the individual glycan profiles of the 2,400 year old “Scythian warrior” and “Scythian princess”, respectively. Compared to the contemporary apartment mummy both Scythian mummies yielded lower total amounts of N-glycans and smaller structures. In addition, although these mummies are approximately of the same age, the “Scythian princess” sample yielded significantly less glycans (in numbers and abundances) than the “Scythian warrior”. Finally, the



**Figure 2** | Representative EICs of abundant N-glycans isolated from a) 4 years old apartment mummy, b) 2,400 years old Scythian warrior mummy-B, c) 2,400 years old Scythian princess mummy-A, and d) 5,300 years old iceman mummy. Circle (white), hexose; circle (green), mannose; square (white), N-acetyl hexosamine; square (blue), N-acetyl glucosamine; triangle (red), fucose; diamond (purple), N-acetyl neuraminic acid.

oldest natural ice mummy, “The Iceman”, yielded the least number of structures and abundances as shown in Figure 2d. Only thirteen N-glycan structures were detected.

In general, the older the sample the fewer glycans observed, with one exception. The “Scythian warrior” sample contained almost as many compounds as the fresh tissue and nearly the same number of compositions as the apartment sample (Table 2). We may speculate that the “Scythian warrior”’s body was better preserved than that of the “Scythian princess”<sup>7,13</sup> therefore loss of structures may not be strictly associated with the age of the sample. This might lead us to hypothesize that the “Scythian princess” died further away from the burial site than the “Scythian warrior” and/or that there was a seasonal differences in the time of death, as the ambient temperature has a very high influence on the putrefaction processes.

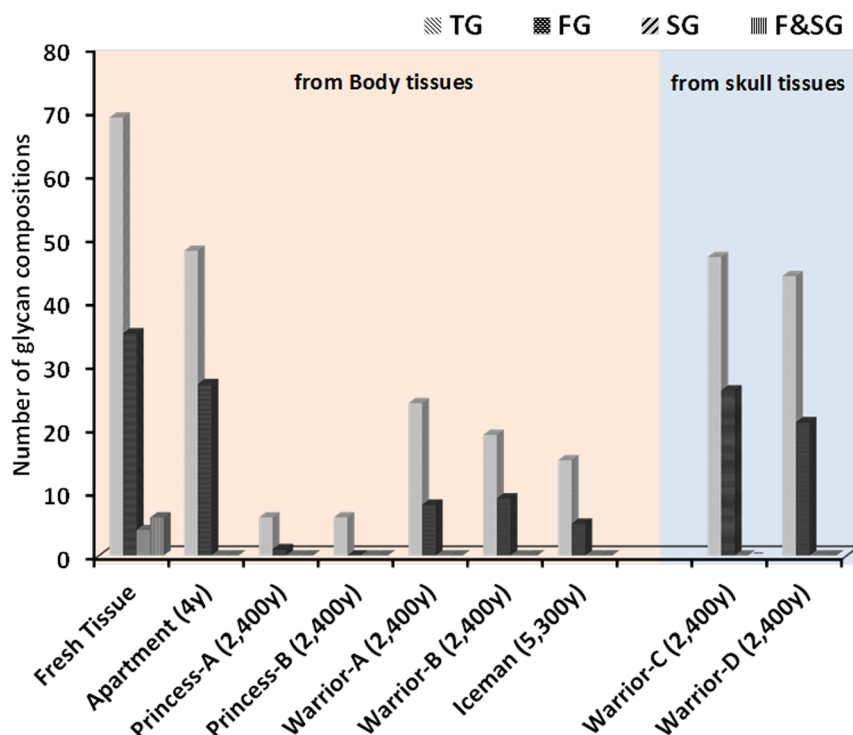
Figure 3 summarizes the number of glycan compositions found in the fresh tissue and the tissue samples from the four natural mummies. We found that mummy age was inversely proportional to the number of N-glycan structures. In particular, the number of N-glycans observed in the Iceman was significantly less compared to the other mummies. Forty-eight N-glycans were detected in the 4 year old apartment mummy, while only thirteen N-glycans were detected in the 5,300 year old Iceman (a 73% decrease). In addition to the overall glycan profile, fucosylation and sialylation were also compared at different time points (Figure 3). Sialylated glycans were only observed in fresh tissue. This observation suggests that sialic acid is easily degraded (or removed) over time in a natural environment. When comparing the newest mummy sample (4 year old apartment mummy) with fresh tissue, there is also a noticeable decrease in the number of fucosylated glycans found but the overall trend shows that fucosylation is relatively stable in a natural environment over time compared to sialylation. Even more interestingly, the “Scythian war-

rior” “C” and “D” samples have a relatively high number of N-glycan structures. The “C” sample is from his skull while sample “D” was extracted from within the inner walls of his mouth. This result clearly illustrates that glycan degradation is strongly correlated with the degree of hydration and dehydration. In addition, all N-glycans found in the various mummies were also observed in fresh human tissue, but we also found several unique N-glycans that are solely present in the mouth tissue samples, independent of age. These are highly fucosylated N-glycans consisting of [Hex]<sub>4-7</sub>[HexNAc]<sub>3-6</sub>[F]<sub>3-6</sub>. A high degree of fucosylation is a characteristic for N-glycan structures in living human saliva<sup>30</sup> which correlates to mummy mouth microbiota.

**Structural confirmation of N-glycans by tandem MS.** The glycan compositions listed in Supplemental Table 1 were determined based on accurate masses, with the more abundant species confirmed further by tandem MS. A putative structure may be deduced from glycan composition because all N-linked glycans share a common trimannosyl core structure consisting of mannoses and GlcNAc residues (Man<sub>3</sub>GlcNAc<sub>2</sub>). Representative MS/MS spectra of N-glycans corresponding to high mannose type N-glycan (Man<sub>7</sub>GlcNAc<sub>2</sub>) and core fucosylated glycan (Man<sub>3</sub>GlcNAc<sub>2</sub>F<sub>1</sub>) are shown in Supplemental Figure 2. Extensive fragmentation was observed in a single MS/MS event. The glycosidic bond cleavages (Supplemental Figure 2a) were observed along with fragments corresponding to subsequent mannose losses (i.e., *m/z* 1396.5, 1234.4, 1072.4, 910.3, 748.3, 586.2). The ion at *m/z* 486.1 corresponds to the trimannosyl core (Man<sub>3</sub>).

A unique mono-fucosylated N-glycan structure is shown in Supplemental Figure 2b. The series loss of fucose and HexNAc from precursor ion *m/z* 1057.39 yielding *m/z* 689.25 suggests that fucose is





**Figure 3** | Comparison with the number of N-glycan compositions found in the fresh tissue and four natural mummies including various tissues. The number of fucosylated and sialylated N-glycans in each mummy samples also were compared with total glycan. TG, total glycan; FG, fucosylated glycan; SG, sialylated glycan; F&SG, fucosylated and sialylated glycan.

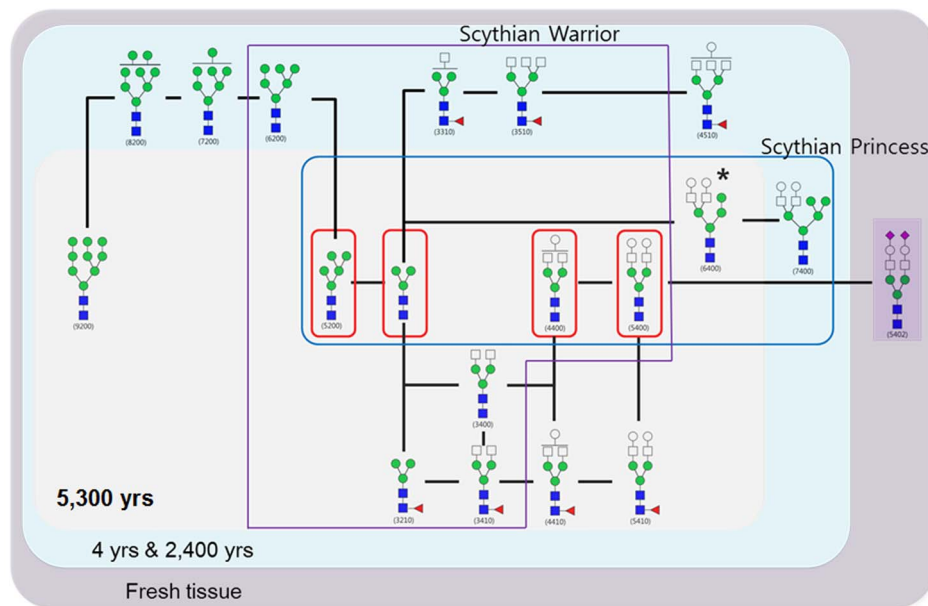
bound to HexNAc located at the reducing end. In addition, there is no direct loss of the HexNAc reducing end (221 Da) from precursor, confirming the position of the fucose. Finally, the fragmentation patterns observed in the tandem mass spectra provide substantial information for the structural elucidation of glycans.

**Variations in the glycosylation of mummies.** We found distinct changes in the N-glycosylation of human glycoproteins in terms of sialylation, fucosylation, and glycan branching (antenna) with the age of the mummy. In general, the number of glycans and their size dramatically decreases with increasing time. Interestingly, sialylated glycans were not observed in any ancient mummies while fucosylated N-glycans were found in all mummies, although the relative abundance of the fucosylated N-glycans was decreased with the mummy's age. Again, while complex-type fucosylated or sialylated N-glycans were abundant in fresh human tissue and in the 4 year old apartment mummy, relatively smaller and high mannose type glycans form the majority of N-glycans in the ancient mummies. Finally, the world's oldest natural ice mummy, the 5,300 year old Iceman, showed a considerably weak glycan profile mainly consisting of the N-glycan core and high mannose-type glycans. A glycan map was constructed to illustrate the general trend in the alteration of glycosylation in mummies using the abundant glycans found in each mummy. Major N-glycans, accounting for more than 95% of the total ion abundance, were selected and their connections examined to explore the biological correlation between glycans found in the four natural human mummies. All glycans were found to be correlated as shown in Figure 4. Each color block represents a different mummy. Major N-glycans from the 5,300 years old Iceman mummy were also present in other mummies. Similarly, every major N-glycan structure identified in the 2,400 year old mummies was also observed in the 4 year old apartment mummy.

At this time, we can only speculate as to why fucose or sialic acid residues were removed from the terminal positions of N-glycan

structures. One possibility is that environmental factors (such as those that affect sample storage and sampling conditions), the person's general health and perhaps even the sex all have significant effects on the level of N-glycosylation in the glycoproteins of the mummies. There have been many hypotheses over the past 20 years as to why the Iceman's body was preserved so effectively. One theory suggests that he must have been covered by snow in the immediate aftermath of his death and subsequently enclosed in glacial ice<sup>31</sup>. However, paleoclimatic data casts some doubt on this particular assumption as during the ensuing warm phases, the ice in the gully may have melted ([www.iceman.it](http://www.iceman.it))<sup>32</sup>. Another theory is that his body was mummified by warm winds due to paleoclimatic changes.

In both these hypotheses for mummification, there is the likely possibility that bacterial exposure may have occurred due to warm winds which is something we have to account for in our conclusions. This theory is supported by previous research done on the Iceman<sup>33</sup>. In general, microbial contributions to the degradation of N-glycans are plausible. Several pathogens as well as intestinal commensal bacteria have the potential to degrade these oligosaccharides<sup>34,35</sup>. Among several bacteria found in the different mummy tissues, *Clostridium aldicarnis* was predominantly found in muscle samples of the Iceman<sup>36</sup>, *Burkholderia pickettii* was found in the stomach and colon, and *C. perfringens*, *C. ghonii*, *C. sordellii*, *Eubacterium tenue*, and *Bacteroides* sp. were found in the colon of the Iceman<sup>37</sup>. The glycolytic potential of these microorganisms is hidden since they have barely been studied and the specific strains present were not sequenced. However, several genomes of different strains of *C. perfringens* are available, and a search on the Integrated Microbial Genome database<sup>38</sup> indicated that sequenced genomes of this species contain several copies of endo- $\beta$ -N-acetylglucosaminidases (EC 3.2.1.96),  $\alpha$ -L-fucosidases (EC 3.2.1.51) and  $\alpha$ -sialidases (EC 3.2.1.18). Also, *Bacteroides* species in general have a substantial glycolytic potential for degrading complex oligosaccharides<sup>39</sup>. On the other hand, little is known about their enzymatic activities in frozen mummies. At this point, we also have no clear understanding of



**Figure 4 | Biological map of major N-glycans which are occupied more than 95% of total intensity from mummy samples.** Glycans appeared in sky blue, and gray area were released apart mummy (4 y) & both Scythian mummy (2,400 y), and iceman mummy (5,300 y) respectively. The glycans that are in purple area are corresponded to fresh tissue. Circle (white), hexose; circle (green), mannose; square (white), N-acetyl hexosamine; square (blue), N-acetyl glucosamine; triangle (red), fucose; diamond (purple), N-acetyl neuraminic acid.

when the human glycosylation machinery was fully developed and the genes for e.g. sialyl transferases were activated in the human genome.

While we are able to speculate on the underlying reasons behind our findings from the Iceman due to the extensive amount of prior research, there is little information available for the “Scythian princess” and the “Scythian warrior”. The differences between the glycomes of these two mummies may be a result of various factors including environmental conditions, health status before death, or sex. In addition the body of the “Scythian princess” had decomposed to a far greater extent than that of the “Scythian warrior” mummy.

## Conclusion

In this study we discovered the world’s oldest N-glycan structures isolated from N-glycoproteins still present in naturally mummified human bodies. Our results clearly demonstrate that N-glycans have been preserved for over thousands of years. Moreover, compositional N-glycan analysis showed some correlation between the mummies and their estimated age. Although the sample size was limited, we were able to characterize the N-glycans but not the complete glycoproteins. It is also important to stress that this study focuses only on the presence or absence of glycans in various mummy tissue samples of different age. There are many parameters affecting the glycomes of mummy specimens, such as sampling differences and environmental conditions, but this study provides a general understanding of mummy tissue glycosylation. The presented findings may lead to future studies using glycomics as a tool for understanding the evolution of both health and the progression of disease.

Recently several researchers have used analysis of microbial successions within postmortem animal samples<sup>40,41</sup>. Our analysis of the world’s oldest glycan specimens promises to open new discussions on the protein associated modifications. The most obvious trend in the N-glycosylation of glycoproteins that are detectable in mummies is the variation over time. The total number of glycans, glycan size (degree of polymerization), and glycan complexity (sialic acid and antenna) decreased with time. Bacterial and/or autolytic degradation and environmental conditions (including sample storage conditions) are both major factors that likely influenced the observed degradation

and preservation of glycans. Based on the available information it might also be possible to speculate that the glycosylation machinery was not fully developed in humans that lived more than 2,000 years ago or, more likely, that other lifestyle factors had an influence on the glycosylation.

## Methods

**Case studies and sampling.** All experiments including mummies and fresh human tissues were performed in accordance with Internal Review Board (IRB) approved protocol from UC Davis Medical Center Clinical Laboratories. And informed consent was obtained for the fresh human tissue sample donation. The sample IDs, sex, age, and estimated age of the corpse till sampling is summarized in Table 1. All samples were cut by a scalpel and then stored in a vessel at  $-20^{\circ}\text{C}$  or below till samples were finally analyzed.

**Iceman.** A small skin sample from Iceman’s left thigh, made available due to damage incurred by a jackhammer during recovery<sup>42</sup>, and stored under argon. With the epidermis lost the outer side of the skin appeared leathery. The inner side contained traces of subcutaneous fat.

**Scythian mummies.** In the 5th Century B.C. the Scythian world was reaching from the Black Sea eastwards to the European and Asian steps including the south of today’s Siberia. Greek historical Herodotus described the horsemen as fierce warriors that were carrying the deceased on wagons to sacred burial grounds. In the Altay Mountains are thousands of burials. The combination of climate and construction of the burials resulted in a block of ice surrounding the corpses. In addition the bodies were prepared for mummification by desiccation. Finally, those mummies can be seen as a combination of natural and artificial mummification.

**Scythian princess (or ice maiden or princess of Ukok).** The mummy was found in a Kurgan barrow discovered in Russia’s Ukok Plateau, at the southern border of Siberia, at 2,300 m above sea level, with China to the south, Mongolia to the east and Kazakhstan to the west. Prior to the burial her brain and other organs have been replaced by fur and wool. The eyeballs e.g. were replaced by fur. In addition the corpse was packed with material like peat and bark containing natural preservatives, e.g. tannin. It is estimated that the burial has taken place at the second half of June. Water could enter earlier but it probably has taken till winter until the tomb was finally frozen with a high probability that it kept being frozen till the mummy was discovered. If the observed putrefaction did not take place during the travel to the burial site there was enough time for the putrefaction in the grave.

**Scythian warrior (or the horseman).** The mummy has several similarities to the Scythian Princess like the frozen tomb being discovered in a Kurgan barrow in Russia’s Ukok Plateau, the corpse being similarly treated to support mummification and the impressive tattoos found on the preserved skin. The Scythian Warrior seemed



to be in a better shape than the Scythian Princess as it was suffering less from putrefaction. So probably the corpse was travelling less long to the burial site and/or death occurred closer to the winter season.

**Apartment mummy.** The mummy is a so-called apartment mummy, one of those cases where a quite isolated and ill individual died in his bed. In the case investigated it did take four years until the corpse was discovered. Besides signs of putrefaction and damages caused by maggots the corpse was well preserved by desiccation. In contrast to the samples of the much older cases the samples still smelled and part of the odor was rancid. In addition the sample was leaving fatty signs when put on paper. So the mummification and/or decay processes were still in progress to a certain extent. The subcutaneous fat sample with dermis was taken from the thigh.

**Fresh tissue.** A soft fresh tissue taken from the right posterior thigh of a 38-year-old white male.

**Rehydration.** Tissues from the 5,300 year old Iceman (Oetzi), the 2400 year old Scythian princess, the 2,400 year old Scythian warrior, and a 4 year old apartment mummy were used for this study. First, all mummy tissues were rehydrated with a mixture of  $\text{Na}_2\text{CO}_3$ : ethanol: water (2:3:5, v:v:v) for 36 hours. Pellets and supernatants were separated and analyzed individually.

A soft fresh tissue was used to compare overall glycosylation with mummy tissues. Because fresh tissue sample was not as dry as mummy samples, rehydration process was by-passed and glycans were directly released from tissue after homogenization.

**Glycan release.** PNGase F-based enzymatic release was used for N-glycan analysis and O-glycans were chemically released by beta-elimination using sodium hydroxide and additionally sodium borohydride to prevent degradation of glycans. All glycans were readily purified and enriched using a graphitized carbon (PGC) cartridge. Accordingly, the N and O-glycans were analyzed by microfluidic chip-based nano-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry (HPLC-Chip/Q-TOF MS). Structural elucidation of abundant N-glycans found in mummy tissues was performed using tandem MS.

**N-glycan analysis by mass spectrometry.** Fractionated and enriched glycans were analyzed using an Agilent 1200 series LC system connected to an Agilent 6520 Q-TOF MS (Agilent Technologies, Santa Clara, CA). The HPLC-Chip/Q-TOF system was equipped with a micro well-plate auto sampler (maintained at 6°C), a capillary loading pump for sample enrichment, a nano flow pump as the analytical pump for sample separation, HPLC-Chip Cube, and the Agilent 6520 Q-TOF MS detector. Data dependent tandem data obtained following LC separation on the microfluidic chip consisting of a 4 mm  $\times$  0.040 mm i.d. enrichment column and a 43 mm  $\times$  0.075 mm i.d. analytical column, both packed with 5  $\mu\text{m}$  porous graphitized carbon (PGC) as stationary phase. LC separation was yielded by using binary gradient solvent A, 3.0% acetonitrile/water (v/v) with 0.1% formic acid and B, 90% acetonitrile/water (v/v) with 0.1% formic acid. A flow rate of 4  $\mu\text{L}/\text{min}$  of solvent A was used for sample loading with 2  $\mu\text{L}$  injection volume. Gradient separation was performed using 0% B (0.00–2.50 min), 0 to 16% B (2.50–20.00 min), 16 to 44% B (20.00–30.00 min), 44 to 100% B (30.00–35.00 min), and 100% B (35.00–45.00 min). The drying gas temperature was set to 325°C with a flow rate of 4 L/min. MS and MS/MS spectra were obtained in the positive ionization mode with an acquisition time of 1587 milliseconds per spectrum and acquisition rate of 0.63 spectra per second. Tandem MS obtained by collision induced fragmentation with nitrogen as the collision gas using a series of collision energies that were dependent on the  $m/z$  values of the different glycans. The collision energies correspond to voltages ( $V_{\text{collision}}$ ) that were based on the equation:  $V_{\text{collision}} = m/z (1.8/100 \text{ Da}) \text{ volts} - 2.4 \text{ volts}$ , where the slope and offset of the voltages were set at (1.8/100 Da) and (−2.4) respectively.

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## Acknowledgments

We are grateful for the support provided by the National Research Foundation (NRF-2013M3A9B6075933) as well as the Converging Research Center Program via the Ministry of Science, ICT and Future Planning, Korea (2013K000430 for H.J. An), and the National Institutes of Health (RO1GM049077 for C.B.L.). We also thank Horst Seidler and Eberhard Lorbeer for support to access the Iceman sample and Natalie Polosmak, Horst Seidler, Kurt J. Irgolic, Walter Gössler for support to access the samples of the Skythian mummies. Finally we would like to thank to Jong-Gon Kim (Agilent Technologies, Inc) for instrument and technical support.

## Author contributions

All authors contributed extensively to the work presented in this paper. R.G., T.B. and C.R. collected the samples, S.O., H.J.A., R.G. and C.B.L. designed the experiment, S.O., B.J.K.,

G.R., J.H.K. and L.M.D. performed the experiment and conducted data analysis. S.O., B.J.K., H.J.A., R.G. and C.B.L. discussed experimental results. R.G., J.H.K., D.G. and D.A.M. described microbial and evolutionary perspective of the findings. All authors discussed the results and implications and commented on the manuscript at all stages. S.O. and B.J.K. contributed equally to this work as co-first authors.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Ozcan, S. *et al.* Glycosylated proteins preserved over millennia: N-glycan analysis of Tyrolean Iceman, Scythian Princess and Warrior. *Sci. Rep.* **4**, 4963; DOI:10.1038/srep04963 (2014).



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